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Accelerated Heterologous Adenovirus Prime-Boost SIV Vaccine in Neonatal Rhesus Monkeys

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A pediatric human immunodeficiency virus type 1 (HIV-1) vaccine would be desirable to protect infants against HIV-1 transmission from breast-feeding. Such a vaccine would need to induce protective immunity at mucosal surfaces in neonates as soon as possible after birth. Recombinant adenovirus (rAd) vectors have been shown to elicit potent systemic and mucosal virus-specific immune responses in adult nonhuman primates and humans, but these vectors have not previously been comprehensively studied in infants. In this study, we demonstrate that a single injection of rAd26 encoding simian immunodeficiency virus mac239 (SIVmac239) Gag on the day of birth elicited detectable Gag-specific cellular immune responses in rhesus monkeys, but these responses were transient and waned quickly. In contrast, an accelerated heterologous prime-boost regimen involving administration of rAd35 at birth and rAd26 at 4 weeks of life elicited potent and durable Gag-specific cellular and humoral immune responses in neonatal rhesus monkeys, including mucosal responses that remained detectable at 1 year of age. These results suggest the potential of an accelerated heterologous rAd prime-boost regimen as a candidate HIV-1 vaccine for newborns.

Human immunodeficiency virus type 1 (HIV-1)-infected women in developing countries typically breast-feed infants for 6 to 12 months, since breast-feeding has been shown to provide overall survival benefits (19). However, breast-feeding remains an important route of HIV-1 transmission and accounts for 33 to 50% of new infant HIV-1 infections worldwide (9, 11, 28, 29). Most breast milk-mediated HIV-1 transmission occurs in the first 6 months of life (10, 28). Interventions are thus needed to prevent breast milk HIV-1 transmission in infants born to HIV-1-infected mothers.

Antiretroviral therapy (ART) has been recommended for HIV-1-infected women during pregnancy and breast-feeding and for infants born to HIV-1-infected mothers (16, 34, 36), but access to effective ART in resource-poor settings still remains limited. Passive immunization of human HIV-1-specific neutralizing serum and monoclonal antibodies (MAbs) has been shown to afford protection against oral exposure to simian-human immunodeficiency virus (SHIV) in neonatal rhesus monkeys (12, 15, 32, 39), but this approach is not practical for widespread use in the developing world. Another approach is the development of a safe and effective HIV-1 vaccine to protect infants from breast milk HIV-1 transmission. A pediatric HIV-1 vaccine would need to elicit potent and durable virus-specific immune responses in both systemic and mucosal immune compartments, including in the oral cavity and gastrointestinal tract, as early as possible after birth.

There are multiple challenges associated with the development of a pediatric HIV-1 vaccine. First, neonates have immature immune systems, and it is unclear whether vaccine vectors that are immunogenic in adults will be comparably immunogenic in neonates. Very few studies of HIV-1 or simian immunodeficiency virus (SIV) vaccines have been performed with neonatal rhesus monkeys (23, 37, 38, 40) and humans (8, 25, 26). Moreover, heterologous prime-boost regimens that have been studied with adult rhesus monkeys (5, 7, 22) typically require 6 months or longer to induce peak immune responses, and such timing would not be optimal for a pediatric HIV-1 vaccine. Thus, novel vaccine regimens need to be developed to induce immune responses rapidly in neonates.

In this study, we tested both single-shot immunization and an accelerated prime-boost regimen with recombinant adenovirus (rAd) vectors in neonatal rhesus monkeys. While a single immunization with rAd26 encoding SIVmac239 Gag at birth elicited detectable Gag-specific immune responses, these responses were transient. In contrast, an accelerated heterologous rAd prime-boost regimen given at birth and 1 month of age elicited potent and durable immune responses in both the systemic and mucosal immune compartments in neonatal rhesus monkeys.

MATERIALS AND METHODS

Rhesus monkeys and immunizations. Newborn specific-pathogen-free rhesus monkeys (*Macaca mulatta*) were delivered and housed at the New England Primate Research Center (Southborough, MA). The presence of the *Mamu-A*01* allele was determined by PCR and sequencing (27). Monkeys were injected intramuscularly (i.m.) with 10^{11} viral particles (vp) of rAd serotype 26 or 35 vectors expressing SIVmac239 Gag in 1 ml of sterile phosphate-buffered saline (PBS) divided equally between the two quadriceps muscles. This study was approved by the Harvard Medical School Institutional Animal Care and Use Committee (IACUC).

Collection and processing of specimens. Peripheral blood was collected to determine systemic Gag-specific cellular and humoral immune responses. At week 52, peripheral lymph nodes, bronchoalveolar lavage (BAL) fluid, and pinch biopsy specimens of colorectal, duodenal, and oral cavity mucosae were collected to evaluate mucosal Gag-specific T lym-

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phocyte responses. IACUC recommendations did not allow biopsies before 1 year of age.

Lymphocytes were isolated from peripheral blood by Ficoll density gradient sedimentation. Plasma was collected and stored at −80°C. Lymph nodes were processed to obtain cell suspension by dissecting them with scalpels in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and passing the cell homogenate through a cell strainer. BAL fluid lymphocytes were collected by centrifuging lavage fluid at 482 × *g* for 5 min. Mucosal lymphocytes were isolated from tissues essentially as previously described (24). Briefly, biopsy specimens were incubated in RPMI 1640 containing 10% FBS supplemented with 200 U/ml of type IV collagenase (Sigma-Aldrich) and 30 U/ml of DNase I (Sigma-Aldrich) at 37°C with rocking for 30 min. The digested biopsy tissues were then homogenized, and the solution was strained with a 70-μm (pore size) cell strainer (BD Falcon). The cell suspension was then centrifuged at 695 × *g* for 25 min on a 35% Percoll (Sigma-Aldrich) gradient. Pellets containing lymphocytes were collected and processed for assays.

ICS assays. Intracellular cytokine staining (ICS) assays were performed essentially as previously described (21, 22). All MAbs were purchased from BD Biosciences unless otherwise indicated. Peripheral blood mononuclear cells (PBMC) (1.5×10^6) were incubated for 6 h at 37°C with medium, 10 pg/ml of phorbol myristate acetate (PMA) and 1 μg/ml of ionomycin (Sigma-Aldrich), or 2 μg/ml of SIVmac239 Gag peptide pool. Cultures contained monensin (GolgiStop; BD Biosciences) and 1 μg/ml of a MAb against human CD49d (clone 9F10; BD Biosciences). The cells were then stained with predetermined titers of MAbs against CD3 (SP34; Alexa 700), CD4 (L200; AmCyan), CD8 (SK1; allophycocyanin-cyanine 7 [APC-Cy7]), CD28 (L293; peridinin chlorophyll-A-cyanine 5.5 [PerCP-Cy5.5]), CD95 (DX2; phycoerythrin [PE]), CD69 (TP1.55.3; phycoerythrin-Texas red [energy-coupled dye; ECD]; Beckman Coulter), gamma interferon (IFN-γ) (B27; phycoerythrin-cyanine 7 [PE-Cy7]), interleukin 2 (IL-2) (MQ1-17H12; allophycocyanin [APC]), and tumor necrosis factor alpha (TNF-α) (Mab11; fluorescein isothiocyanate [FITC]). Stained samples were fixed with 1.0% paraformaldehyde solution and evaluated with an LSR II (BD Biosciences). Flow data were analyzed using FlowJo software (Tree Star). Approximately 300,000 to 500,000 events were collected per sample. Background responses were typically below 0.01% of gated $CD4^+$ or $CD8^+$ T lymphocytes. ICS values were analyzed following subtraction of backgrounds.

Tetramer-binding assays. Multiparameter tetramer-binding assays were performed essentially as previously described (31, 35). PBMC (1×10^6) or mucosal lymphocytes were stained with Mamu-A*01 tetramers labeled with PE and folded around the immunodominant SIV Gag epitope CM9 (CTPYDINQM; also known as p11c) (4) in conjunction with predetermined titers of MAbs against CD3 (SP34.2; Alexa 700), CD4 (L200; AmCyan), CD8 (SK1; APC-Cy7), CD69 (TP1.55.3; ECD; Beckman Coulter), CCR7 (150503; FITC; R&D Systems), CD95 (DX2; APC), and CD28 (L293; PerCP-Cy5.5) at room temperature for 30 min. The background for tetramer staining was typically below 0.01% of gated $CD8^+$ T lymphocytes from peripheral blood and below 0.05% of gated $CD8^+$ T lymphocytes for biopsy samples.

ELISA. Gag-specific humoral immune responses were assessed by an enzyme-linked immunosorbent assay (ELISA) using plasma samples. Briefly, recombinant SIVmac251 p28 protein (MyBioSource) was used to coat a Nunc Immuno ELISA plate overnight. Serially diluted plasma samples were added for an incubation of 1 h and the plate was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Jackson ImmunoResearch Laboratories) and developed with TMB substrate (KPL). The absorbance at a wavelength of 450 nm with that at 550 nm subtracted was recorded and used for analysis.

Adenovirus neutralization assay. Ad26-specific neutralizing antibody (NAb) titers were assessed by luciferase-based virus neutralization assays as described previously (6). A549 human lung carcinoma cells were plated at a density of 1×10^4 cells per well in 96-well plates and infected with a replication-incompetent rAd26-Luc reporter construct (with an E1/E3 deletion) at a multiplicity of infection of 500 with 2-fold serial dilutions of plasma in 200-μl reaction volumes. Following a 24-h incubation, luciferase activity in the cells was measured using the Steady-Glo luciferase reagent system (Promega) with a Victor 1420 multilabel counter (Perkin-Elmer). Neutralization titers were defined as the maximum serum dilution that neutralized 90% of luciferase activity.

Statistical analysis. Comparisons of immunologic data among groups of monkeys were performed by analysis of variance (ANOVA) with Tukey posttest adjustments utilizing GraphPad Prism 4.

RESULTS

Cellular immune subsets in neonatal rhesus monkeys. We initiated studies to assess cellular immune subsets in healthy neonatal rhesus monkeys on the day of birth. Peripheral blood was evaluated for both the numbers and phenotypes of CD4 and CD8 T lymphocytes by multiparameter flow cytometry. Neonatal monkeys had a significantly (2.2-fold) higher CD4/CD8 T lymphocyte ratio than did adult monkeys (Fig. 1A), consistent with prior observations (41). However, by 1 year of age, the CD4/CD8 ratio approached that of adult monkeys (Fig. 1A). At birth, both CD8 and CD4 T lymphocytes exhibited a naïve phenotype ($CD95^-$ $CD28^+$) with minimal effector memory (EM; $CD95^+$ $CD28^-$) and central memory (CM; $CD95^+$ $CD28^+$) cells (30), as expected (Fig. 1B). This phenotype evolved gradually over time and was not significantly impacted by vaccination (Fig. 1B and C).

Single-shot rAd26 immunization elicits detectable but transient Gag-specific immune responses in neonatal rhesus monkeys. We have previously reported the immunogenicity of single-shot rAd immunization in adult rhesus monkeys (20–22), but rAd vectors have not previously been studied using neonatal rhesus monkeys with an immature immune system. To evaluate the immunogenicity of single-shot rAd immunization in neonates, two rhesus monkeys were immunized i.m. with 10^{11} vp rAd26 encoding SIVmac239 Gag on the day of birth. Gag-specific CD8 and CD4 T lymphocyte responses were assessed by multiparameter intracellular cytokine staining (ICS) assays. Gag-specific cellular immune responses were detected at week 4 of life with magnitudes comparable to those observed in adult monkeys (Fig. 2A) (21). However, unlike in adult rhesus monkeys, these cellular immune responses waned quickly, and only marginal CD8 T lymphocyte responses were detected by week 10 (Fig. 2A). These data suggest that rAd26 vectors were immunogenic in neonates but that a single-shot immunization at birth was insufficient to induce durable immune responses. Nevertheless, robust vector-specific NAb titers were induced in the neonatal rhesus monkeys (Fig. 2B) at levels comparable to those observed in adult monkeys (1, 20).

Accelerated heterologous rAd prime-boost regimen elicits potent and durable Gag-specific immune responses in neonatal rhesus monkeys. Heterologous rAd prime-boost vaccine regimens have been shown to elicit potent and durable SIV-specific immune responses in multiple immune compartments in adult rhesus monkeys (7, 20, 22). However, most heterologous prime-boost regimens require 6 months or more to elicit peak immune responses (5, 7, 22). Such a time frame would not be compatible with a pediatric HIV-1 vaccine that needs to induce protective immunity as soon as possible after birth. We therefore tested a novel accelerated heterologous rAd prime-boost regimen in 6 neonatal rhesus monkeys. Monkeys were primed i.m. with 10^{11} vp rAd35 encoding SIVmac239 Gag on the day of birth and were boosted with 10^{11} vp rAd26 encoding the same antigen at week 4

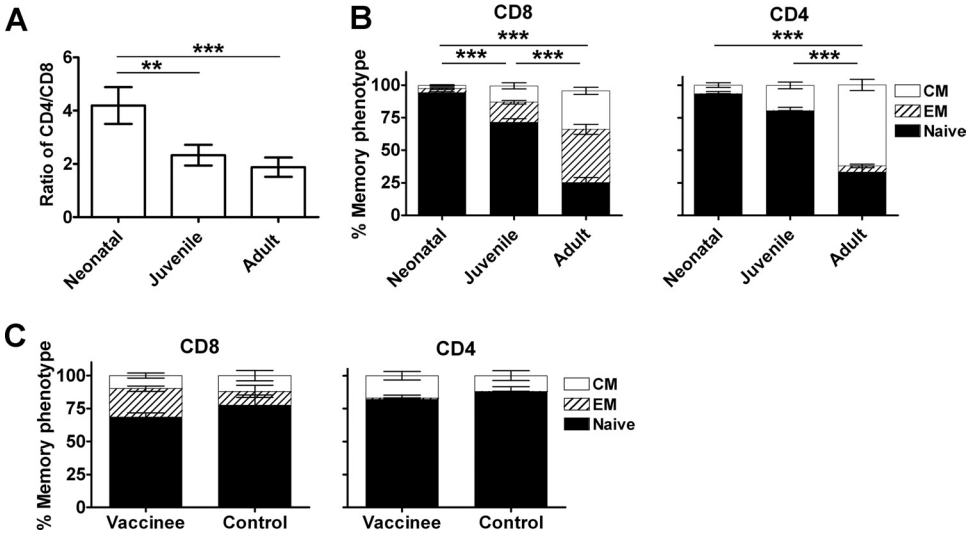

FIG 1 Cellular immune subsets in neonatal rhesus monkeys. PBMC were isolated from peripheral blood of neonatal rhesus monkeys on the day of birth ($n = 9$), juvenile rhesus monkeys at 1 year of age ($n = 9$), and adult rhesus monkeys ($n = 12$). The number and phenotype of CD8 and CD4 T lymphocytes were determined by multiparameter flow cytometry. (A) Ratio of CD4/CD8 T lymphocytes in peripheral blood. **, $P < 0.01$; ***, $P < 0.001$ (ANOVA). (B) Phenotype of CD8 and CD4 T lymphocytes with $CD95^-$ $CD28^+$ defining naïve cells, $CD95^+$ $CD28^-$ defining effector memory (EM) cells, and $CD95^+$ $CD28^+$ defining central memory (CM) cells (30). ***, $P < 0.001$ (ANOVA). (C) Phenotype of CD8 and CD4 T lymphocytes of neonatal monkeys following immunizations with rAd35-rAd26 (vaccinee) or saline (control) at 4 weeks after boost immunization.

of life. These vaccines proved safe and well tolerated in all the neonatal monkeys, without evidence of adverse effects. These monkeys developed Gag-specific cellular immune responses at week 4 (Fig. 3B) with magnitudes similar to those observed in the pilot study (Fig. 2). Importantly, substantially higher levels of Gag-specific CD8 and CD4 T lymphocytes secreting IFN-γ, TNF-α, and/or IL-2 were observed following the boost immunization at week 4, with peak responses at week 8 of life (Fig. 3A and B). Moreover, these responses remained detectable at 1 year of age (Fig. 3B). Particularly high CD8 T lymphocyte responses with an EM phenotype were observed, which may be relevant for a vaccine that needs to protect rapidly against viral challenges (Fig. 3C). These data demonstrate that the accelerated heterologous rAd35-rAd26 prime-boost regimen elicited rapid and potent cellular immune responses in neonatal rhesus monkeys.

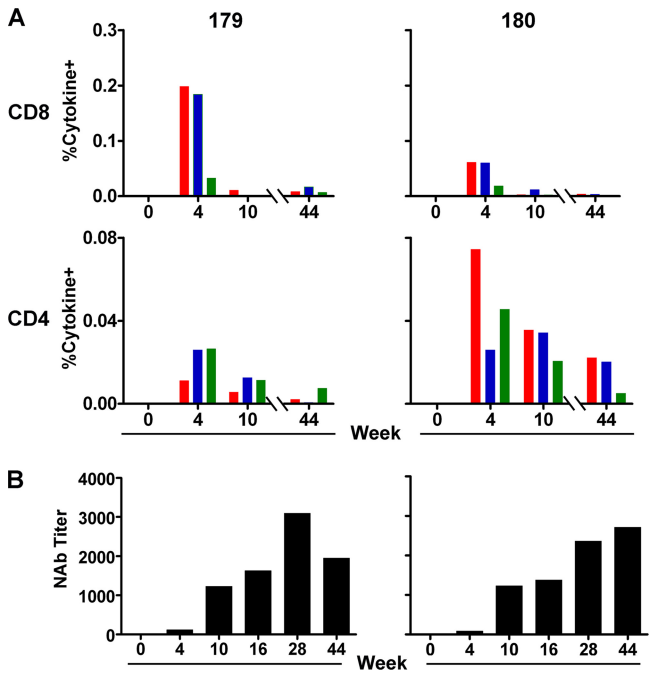

FIG 2 Single-shot rAd26 immunization elicits Gag-specific cellular immune responses in neonatal monkeys. Two neonatal rhesus monkeys (179 and 180) were immunized i.m. with 10^{11} vp rAd26 encoding SIVmac239 Gag on the day of birth. (A) Gag-specific cellular immune responses in both CD8 (upper panels) and CD4 (lower panels) T lymphocyte compartments were evaluated by multiparameter ICS assays. (B) Ad26 NAb titers in plasma were determined by Ad26 neutralization assays.

Gag-specific humoral immune responses were also elicited by this heterologous prime-boost vaccine regimen. Vaccinated monkeys exhibited Gag-specific antibody responses at week 4 of life, and these responses remained detectable at 1 year of age (Fig. 3D), perhaps reflecting the CD4 T lymphocyte help induced by the vaccine. No Gag-specific antibody responses were observed on the day of birth or in unvaccinated age-matched control animals. These data indicate that the accelerated heterologous rAd prime-boost regimen induced rapid and durable cellular and humoral immune responses in neonatal rhesus monkeys.

Phenotypic evolution of Gag-specific cellular immune responses in neonatal rhesus monkeys. Since neonatal monkeys have immature immune systems, we compared the magnitude and phenotype of Gag-specific cellular immune responses elicited by the heterologous rAd35-rAd26 prime-boost regimen in neonatal monkeys and adult monkeys. In a separate study, eight adult rhesus monkeys (3 to 5 years old) received 10^{11} vp rAd35 encoding SIVmac239 Gag, Pol, and Env at week 0 and 10^{11} vp rAd26 encoding the same antigens at week 24 (7). Peak CD8 T lymphocyte responses from neonatal and adult monkeys showed comparable magnitudes and phenotypes in terms of memory subpopulations

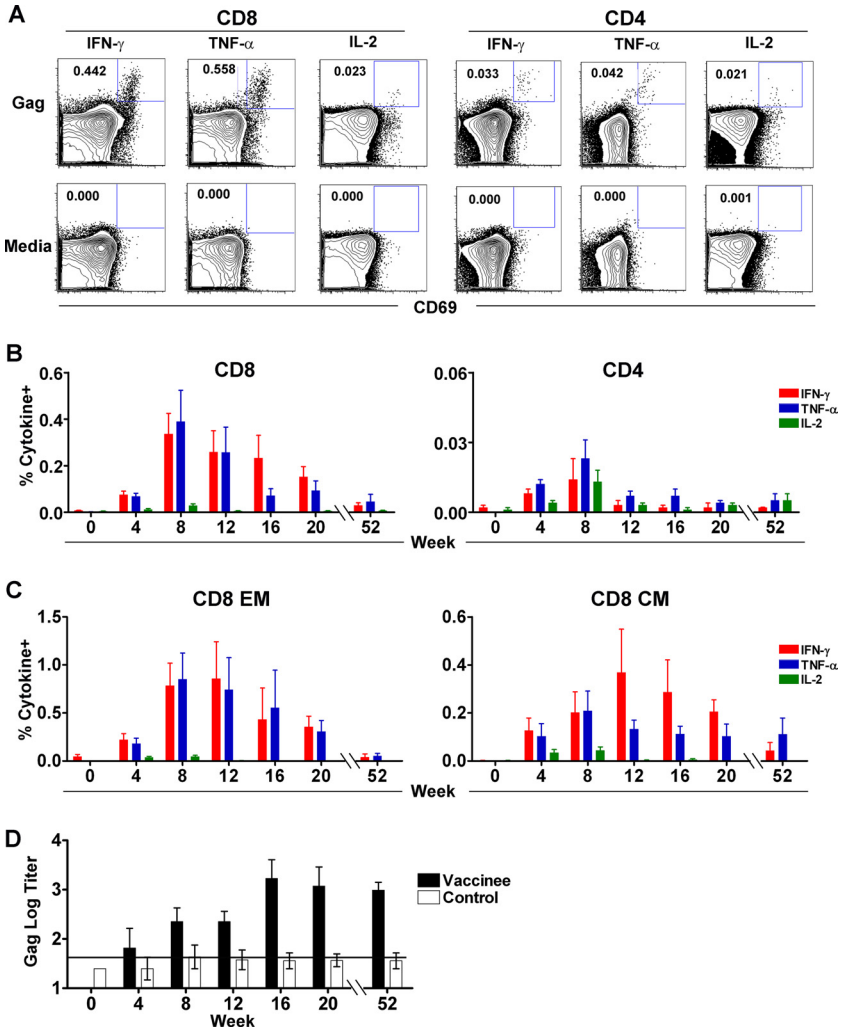

FIG 3 Accelerated heterologous rAd prime-boost regimen elicits potent and durable Gag-specific immune responses in neonatal rhesus monkeys. Six neonatal rhesus monkeys were immunized i.m. with 10^{11} vp rAd35 encoding SIVmac239 Gag on the day of birth and boosted with 10^{11} vp rAd26 encoding the same antigen at week 4 of life. Three neonates received saline injections at the same time points as sham controls. Gag-specific cellular and humoral immune responses were assessed for 1 year. (A) Gag-specific cellular immune responses of a representative vaccinated monkey are shown at week 8. (B) Gag-specific cellular immune responses in the 6 vaccinated neonatal rhesus monkeys. Means ± standard deviations are shown. (C) Magnitudes of Gag-specific CD8 effector memory (EM) and central memory (CM) T lymphocyte responses by multiparameter flow cytometry. (D) Magnitude of Gag-specific humoral immune responses by ELISA.

(Fig. 4A). In contrast, CD4 T lymphocyte responses in neonatal monkeys were lower than in adult monkeys, likely reflecting the higher CD4/CD8 T lymphocyte ratios in neonatal monkeys compared with adult monkeys (Fig. 1A and 4A).

Two of the six neonatal rhesus monkeys that received the accelerated heterologous rAd prime-boost regimen expressed the *Mamu-A*01* allele (27). Gag-specific CD8 T lymphocyte responses in these monkeys were therefore also monitored by multiparameter tetramer-binding assays to assess *Mamu-A*01*-restricted Gag CM9-specific responses (Fig. 4B). The phenotypic evolution of Gag-specific CD8 T lymphocytes in these monkeys was analyzed as we have previously reported (20) by expression of the memory marker CD95, the lymph node homing marker CCR7, and the costimulatory molecule CD28. $CD95^+$ $CCR7^-$ $CD28^+$ defined transitional effector memory (T_{EM1}) cells, $CD95^+$ $CCR7^-$ $CD28^-$ defined effector memory (T_{EM2}) cells, and $CD95^+$ $CCR7^+$ $CD28^+$ defined central memory (T_{CM}) cells (13). Similar to what we have previously described for adult monkeys (20), peripheral Gag-specific CD8 T lymphocytes in neonatal monkeys exhibited rapid phenotypic evolution from T_{EM1} and T_{EM2} to T_{CM} cells following rAd immunization (Fig. 4B).

Accelerated heterologous rAd prime-boost regimen elicits durable mucosal Gag-specific cellular immune responses in neonatal rhesus monkeys. At week 52, biopsy specimens from multiple mucosal tissues, including colon, duodenum, and the oral cavity, as well as BAL fluid were obtained from the *Mamu-A*01*$^+$ monkeys. We were not permitted to perform mucosal biopsies on neonatal monkeys prior to 1 year of age per IACUC recommendations. As expected, mucosal T lymphocytes in these animals were primarily $CD95^+$ memory cells, whereas the majority of lymphocytes from peripheral blood or peripheral lymph nodes were $CD95^-$ naïve cells (Fig. 5A), and most mucosal T

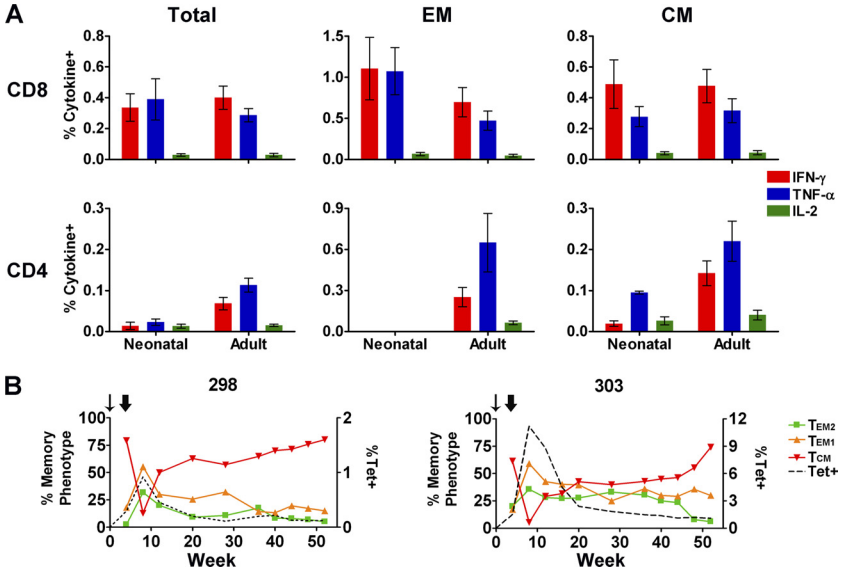

FIG 4 Vaccine-elicited Gag-specific cellular immune responses exhibit similar phenotypic evolution in neonatal and adult monkeys. The magnitudes and phenotypes of vaccine-elicited Gag-specific cellular immune responses at peak in neonatal monkeys were compared with those observed in adult monkeys (3 to 5 years old) in a separate study which received 10^{11} vp of Ad35-Gag/Pol/Env at week 0 and 10^{11} vp of Ad26-Gag/Pol/Env at week 24 (7). (A) Gag-specific cellular immune responses of total CD8 and CD4 T lymphocytes as well as EM and CM compartments in neonatal and adult rhesus monkeys. (B) Gag-specific CD8 T lymphocyte responses in peripheral blood specific for the *Mamu-A*01*-restricted Gag epitope CM9 (CTPYDINQM) in *Mamu-A*01*$^+$ rhesus monkeys 298 and 303 were determined by multiparameter tetramer-binding assays. Phenotypic evolution of Gag-specific (Tet$^+$) CD8 T lymphocyte responses was analyzed by expression of the memory marker CD95, lymph node homing marker CCR7, and the costimulatory molecule CD28. CD95$^+$ CCR7$^-$ CD28$^+$ defined transitional effector memory (T_{EM1}) cells, CD95$^+$ CCR7$^-$ CD28$^-$ defined effector memory (T_{EM2}) cells, and CD95$^+$ CCR7$^+$ CD28$^+$ defined central memory (T_{CM}) cells (20). Thin arrows represent rAd35 priming, and thick arrows represent rAd26 boosting.

lymphocytes were T_{EM1} and T_{EM2} cells (Fig. 5B). Gag-specific CD8 T lymphocyte responses were detected by CM9-specific tetramer-binding assays in all mucosal tissues, including colon, duodenum, oral mucosa, and BAL fluid at 1 year of age (Fig. 5C and D). Moreover, while peripheral Gag-specific CD8 T lymphocytes exhibited a T_{CM}-dominated phenotype at this time point, mucosal Gag-specific CD8 T lymphocytes remained predominantly T_{EM1} cells (Fig. 5D), consistent with what we have reported for adult

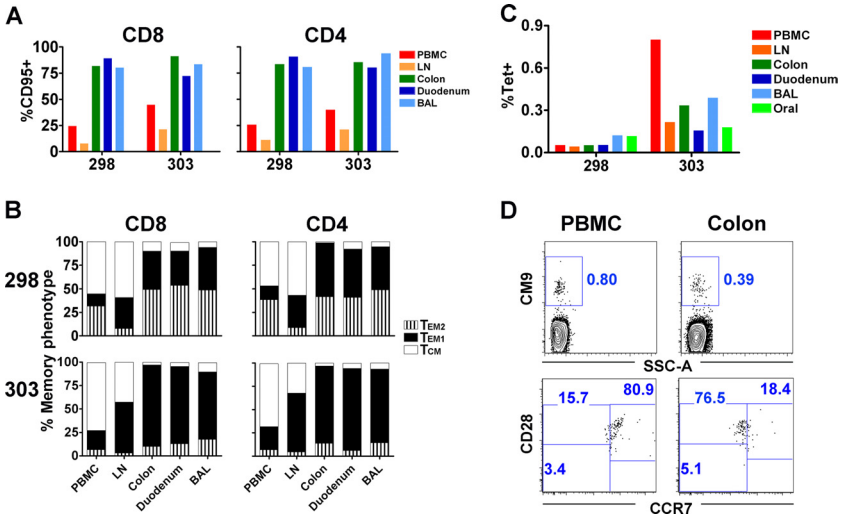

FIG 5 Heterologous rAd prime-boost regimen elicits durable mucosal Gag-specific cellular immune responses in neonatal rhesus monkeys. Peripheral blood, peripheral lymph node (LN), and pinch biopsy specimens from the colon, duodenum, and oral cavity, as well as bronchoalveolar lavage (BAL) fluid, were collected from *Mamu-A*01*$^+$ monkeys 298 and 303 at week 52. The phenotypes of total T lymphocytes and *Mamu-A*01*-restricted Gag epitope CM9 were determined by multiparameter tetramer-binding assays. (A) Percentage of memory (CD95$^+$) CD8 and CD4 T lymphocytes in systemic and mucosal compartments. (B) Phenotype of memory CD8 and CD4 T lymphocytes in systemic and mucosal compartments analyzed by expression of CCR7 and CD28 as described previously (20). (C) Magnitude of Gag CM9-specific (Tet$^+$) CD8 T lymphocytes in systemic and mucosal specimens. (D) Representative data depicting the magnitude and phenotype of systemic (PBMC) and mucosal (colon) Gag CM9-specific (Tet$^+$) CD8 T lymphocyte responses in monkey 303 at week 52. CD95$^+$ CCR7$^-$ CD28$^+$ defined transitional effector memory (T_{EM1}) cells, CD95$^+$ CCR7$^-$ CD28$^-$ defined effector memory (T_{EM2}) cells, and CD95$^+$ CCR7$^+$ CD28$^+$ defined central memory (T_{CM}) cells.

monkeys (20). These data demonstrate that the accelerated heterologous rAd prime-boost regimen elicited durable Gag-specific cellular immune responses in multiple mucosal immune compartments in neonatal rhesus monkeys, including in the oral and gastrointestinal mucosae.

DISCUSSION

Breast milk exposure remains a major route of mother-to-child HIV-1 transmission in the developing world. A neonatal HIV-1 vaccine that rapidly induces systemic and mucosal protective immunity would therefore represent a major advance. A variety of HIV-1 vaccine candidates have been studied with adults, but very few studies of HIV-1 vaccines have been performed in neonates (2, 8, 23, 25, 26, 37, 38, 40). Considering the unique features of the neonatal immune system and the need to induce mucosal immune responses rapidly to protect newborns from breast milk HIV-1 transmission, it is important to investigate the immunogenicity of candidate HIV-1 vaccines directly in neonates. In this study, we have demonstrated that rAd26 and rAd35 vectors (1) are immunogenic in neonatal rhesus monkeys despite the immaturity of the neonatal immune system. A single immunization of rAd26 at birth elicited detectable but transient SIV Gag-specific cellular immune responses. In contrast, an accelerated rAd35-rAd26 prime-boost regimen given at birth and week 4 of life elicited potent and durable cellular and humoral Gag-specific immune responses, including mucosal immune responses in the oral cavity and gastrointestinal tract. These data suggest that further exploration of this strategy as a candidate pediatric HIV-1 vaccine is warranted.

Heterologous rAd prime-boost regimens have been studied extensively in adult rhesus monkeys and typically require 6 months or more to complete the immunization series. It is commonly believed that a late boost is required for the induction of high-magnitude and durable immune responses, but such a lengthy schedule would not be possible for a pediatric HIV-1 vaccine that needs to generate protective immunity as rapidly as possible after birth to protect against ongoing breast milk HIV-1 exposure. In this study, we evaluated a novel accelerated heterologous rAd prime-boost regimen in neonatal monkeys that involved priming with rAd35 vectors at birth and boosting with rAd26 vectors at week 4 of life. This regimen resulted in more potent and durable SIV-specific immune responses and appeared comparable with responses induced in adult monkeys with a substantially longer vaccine regimen. Responses remained detectable at 1 year of age in both systemic and mucosal immune compartments.

In infant rhesus monkeys, the tonsil and intestinal tissues represent the primary sites of viral replication after oral SIV infection (3), and vaccine-elicited SIV-specific IgA in both saliva and plasma was found to be inversely correlated with viral load (23). Therefore, an effective neonatal AIDS vaccine will presumably need to elicit virus-specific immune responses in the oral and gastrointestinal mucosae. We observed durable Gag-specific cellular immune responses at week 52 in mucosal tissues from the oral cavity, colon, and duodenum in monkeys that received the accelerated rAd35-rAd26 prime-boost regimen, despite the systemic intramuscular route of vaccination (18, 20). Moreover, these mucosal Gag-specific T lymphocytes exhibited a T_{EM1}-dominated phenotype, suggesting that these memory T lymphocytes may be able to respond rapidly to viral challenges (17, 33). Additional studies will be required, however, to define the protective efficacy of these vaccine-elicited immune responses against oral viral challenges. Of note, it has recently been reported that vaccine-elicited antibody responses correlated with reduced HIV-1 acquisition risk in the RV144 clinical trial (14).

Our data confirm and extend prior SIV vaccine studies with neonatal rhesus monkeys. It has previously been shown that modified vaccine virus Ankara (MVA) vectors encoding SIV Gag, Pol, and Env administered to neonatal rhesus monkeys at 0 and 3 weeks of age induced virus-specific antibody but not T lymphocyte responses and resulted in lower viral loads than in control monkeys following oral SIVmac251 challenges (40). Both ALVAC and MVA vectors expressing SIV antigens administered to infant rhesus monkeys afforded partial protection against repeated low-dose exposures of SIVmac251, but detailed immunologic characteristics of the vaccine-elicited immune responses were not reported (38). Similarly, vesicular stomatitis virus (VSV) vectors have been tested alone and in combination with MVA vectors in this model (23, 37). Our data with rAd vectors similarly show that viral vaccine vectors are immunogenic in neonates. Our studies further describe the detailed kinetics, phenotype, and functionality of vaccine-elicited cellular immune responses in this model. Multiparameter flow cytometry techniques were critical for generating substantial amounts of immunologic data using limited numbers of cells obtained from the small blood volumes that could be obtained safely from neonatal rhesus monkeys.

In summary, our data demonstrate that a novel accelerated heterologous rAd prime-boost regimen was safe and induced potent and durable Gag-specific cellular and humoral immune responses in both systemic and mucosal immune compartments in neonatal rhesus monkeys. These features may be relevant to the design of a pediatric HIV-1 vaccine to protect against breast milk HIV-1 transmission. However, further investigation is required to compare heterologous and homologous vector regimens and to assess the protective efficacy of these vaccine candidates against viral challenges.

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